DEVELOPMENTAL BIOLOGY 167, 617–620 (1995)

RAPID COMMUNICATION

FGF Can Induce Outgrowth of Somatic Mesoderm both Inside and Outside of Limb-Forming Regions

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Accepted October 19, 1994

In the vertebrate embryo, only somatopleural cells in the limb-forming region are released from the mesodermal layer and undergo outgrowth from the embryonic body to form the limb bud. Molecular signals which regulate limb bud induction are unknown to date. In the present study we examined the ability of fibroblast growth factor (FGF) to induce limb bud formation in chicken embryos. A replication-defective retrovirus encoding FGF type 4 with a reporter, bacterial β-galactosidase, was microinjected into lateral plate mesoderm inside and outside limb-forming regions. Effects of the ectopic and precocious expression of FGF were assessed at various stages after infection. Here we report that somatic mesodermal cells in both flank and limb-forming regions can respond to FGF and induce limb bud-like outgrowth. The supernumerary limb bud induced within a limb-forming region differentiated into extralimb structures. These results strongly suggest potential roles of FGF signaling for induction of limb bud formation.

INTRODUCTION

The first specialization of limb-forming cells is evident when mesenchymal cells are released from the somatic layer of lateral plate mesoderm. These cells migrate laterally and accumulate under the epidermal tissue as a circular bulge to form the limb bud. Thus, although somatopleural mesenchyme stretches from anterior to posterior along the entire length of the embryonic body, only the mesenchymal cells in regions of prospective fore- and hind-limb formation undergo sprouting from the embryonic body to generate the limb bud. The factor(s) which defines regions that induce the limb bud has not been identified. Previous studies on signal networks regulating pattern formation in the already growing limb bud (reviewed by Tabin, 1991) have demonstrated that fibroblast growth factors (FGFs) keep limb mesenchymal cells proliferative and undifferentiated to complete limb outgrowth and patterning (Niswander *et al.*, 1993; Fallon *et al.*, 1994). However, it is unknown whether FGF signaling is involved in the early events of limb bud induction and, if it is, when the somatic mesodermal cells inducing limb bud formation become susceptible to FGF signaling. To determine whether FGF can function in the early induction of mesodermal outgrowth in a restricted manner, both regionally and temporally, we generated a replication-defective retrovirus to coexpress FGF4 and the reporter gene bacterial β-galactosidase. We infected somatic mesodermal cells both inside and outside limb-forming regions with the virus and assessed cellular responses to the precocious introduction of FGF ligand. Cells expressing exogenous FGF gave rise to limb bud-like outgrowth in both flank and limb-forming regions. Furthermore, the supernumerary limb bud induced in limb-forming regions differentiated into extralimb structures including duplicated skeletal elements. These data suggest that FGF signaling may play important roles for limb bud induction in addition to its functions in growing limbs (Niswander *et al.*, 1993; Fallon *et al.*, 1994).

MATERIALS AND METHODS

Virus and in ovo injection. A 0.87-kb fragment containing FGF4 (formerly *hst1*) was isolated from pKOc1 (Taira *et al.*, 1987) by partial digestion with *Ava*II and *Hin*dIII, blunt-ended, and ligated upstream of lacZ in pSNZ (Mikawa *et al.*, 1992), a vector plasmid for a replication-defective variant of the avian spleen necrosis virus (SNV; Dougherty and Temin, 1986). To obtain translation of both FGF4 and β-galactosidase from the dicistronic construct in infected cells, the viral vector was engineered to encode a 5′ flanking sequence of picornavirus between two genes to generate an 5′ cap-inde-

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0012-1606/95 $6.00

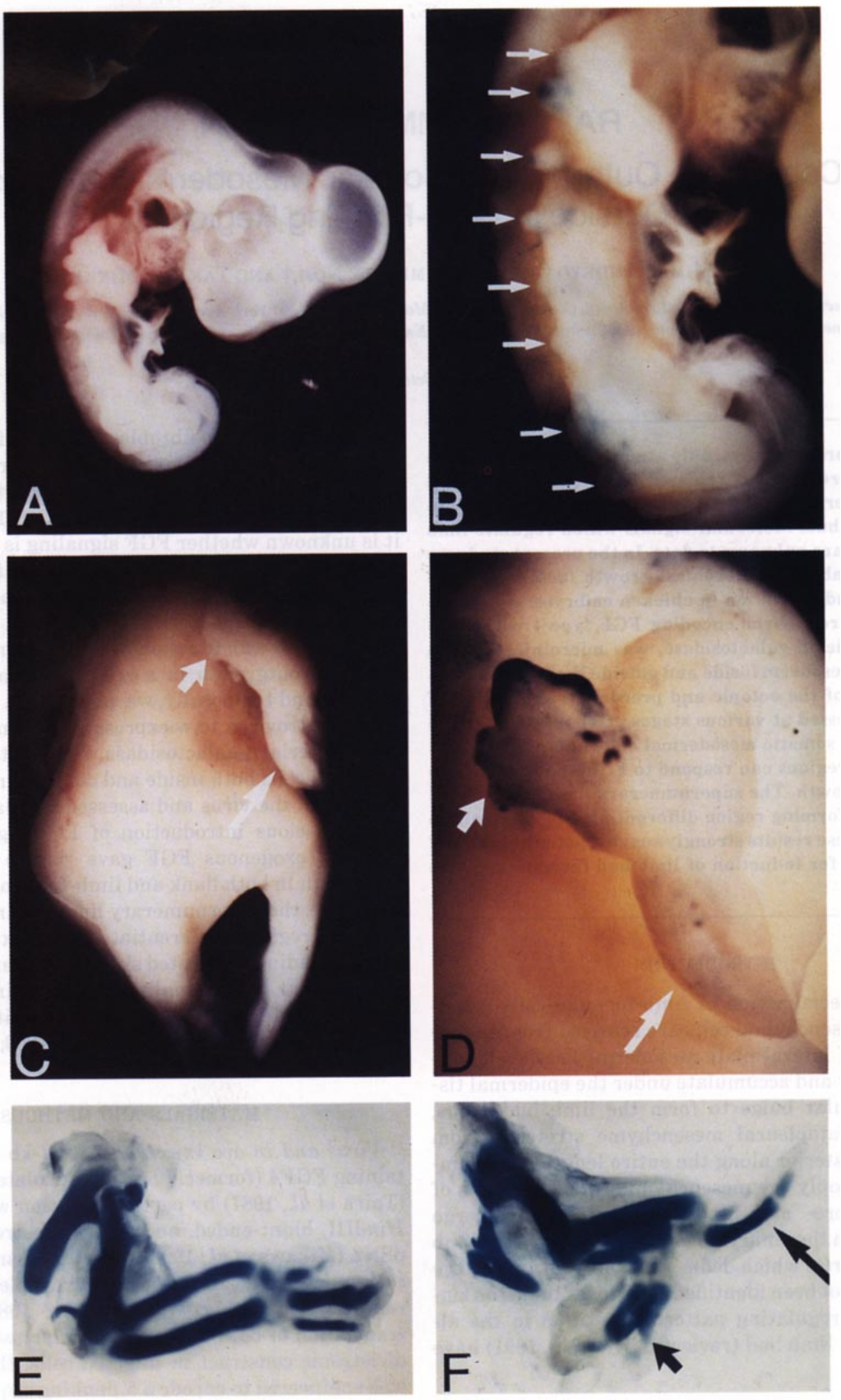
A
B
C
D
E
F

TABLE 1
FREQUENCY OF FGF-DEPENDENT OUTGROWTH ALONG THE EMBRYONIC BODY AXIS

Positions (somite level) and stages of infection	Induction of protrusions	
	Stages 25–27	Stages 33–35
Head (somite level 5–10)		
Stages 13–15	0/19	0/12
Stages 17–18	0/25	0/11
Wing (somite level 15–20)		
Stages 13–15	28/33	17/24
Stages 17–18	3/12	1/9
Flank (somite level 21–25)		
Stages 15–18	21/24	0/20
Leg (somite level 26–32)		
Stages 17–18	15/20	8/17
Posterior to leg		
Stages 17–18	7/12	0/18

Note. Lateral mesoderm tissues were infected with virus encoding FGF4 at HH stages 13–18 depending on the targeted regions. Injection of virus and culture of infected embryos were described in Fig. 1. Extra structures protruding from the embryonic body were examined at two developmental stages (HH stages 25–27 and 33–35). The number of embryos exhibiting extra protrusions in the total number of infected embryos are presented.

pendent internal ribosome entry site (Ghattas *et al.*, 1991). Virus was propagated as described (Mikawa *et al.*, 1992). The virus stock of 10^5–10^6 active virions per 1 ml was usually concentrated approximately 100-fold by centrifugation (Mikawa *et al.*, 1992). Procedures of *in ovo* infection and culture of infected embryos were described previously (Mikawa *et al.*, 1992).

Morphological observations. Procedures for the whole mount staining of β-galactosidase with X-gal were described previously (Mikawa *et al.*, 1992). The cartilage components were visualized after fixation with 5% trichloroacetic acid overnight and incubation with Alcian blue at 75 mg/liter in acid alcohol made of 75% ethanol and 25% glacial acetic acid for 1–2 days. The embryos were gradually dehydrated with ethanol followed by clearing with methyl salicylate.

RESULTS AND DISCUSSION

The concentrated virus stock of 1–5 nl was injected *in ovo* to target a subset of somatic mesodermal cells of the early chicken embryo at HH stages 15–17. When the infected embryos were examined at HH stages 25–27, we often found ectopic structures protruding from the infected region, irrespective of whether the infected tissue was in limb bud-forming regions or in flanking somatic mesoderm (Fig. 1A and Table 1). X-gal staining showed the infected cells dispersed within the developing ectopic structure (Fig. 1B). These results indicate that somatic mesodermal cells even outside of the limb-forming regions can respond to exogenous FGF and are capable of forming an outgrowth from the body wall. However, protrusions were not induced when the same virus was introduced into the somite, indicating that other mesenchymal tissues such as the somites do not undergo outgrowth under the same conditions. Thus, among mesodermal subpopulations, somatic mesoderm is specialized to undergo outgrowth to form the protrusions when exposed to FGF signaling. Later in development, the protrusions induced within limb-forming region differentiated into extralimb structures (Table 1). Figure 1C demonstrates an embryo exhibiting a duplicated wing. X-gal staining revealed that cells expressing transgenes were predominantly localized at apical regions in the extra wing (Fig. 1D). We then examined skeletal elements in the extra wing (Fig. 1F) as well as in the uninfected control wing (Fig. 1E). In most cases, we observed more skeletal elements (Fig. 1F) than Riley *et al.* (1993). In contrast, protrusions induced in the flank regions merged with body wall later in development and in no case differentiated into limb-like structures or affected rib components (Table 1).

Since it has been shown that FGF signaling regulates limb outgrowth and patterning (Niswander *et al.*, 1993; Fallon *et al.*, 1994), we originally hypothesized that only in the limb-forming region are somatopleural cells susceptible to FGF signaling. Our data, however, demonstrated that cells in the somatic layer of the flank or limb-forming regions can induce an outgrowth protruding from the embryonic body when exposed to FGF. These results suggest that regions inducing outgrowth of limb bud may be defined by restricting FGF expression to that region rather than by localizing susceptibility to the signaling. Although it is unclear which member(s) of the FGF family of genes displays expression

FIG. 1. FGF-dependent outgrowth of somatic mesodermal cells inside and outside the limb-forming field. (A) Virus encoding FGF4 was injected into the lateral mesoderm adjacent to alternating somites of a HH stage 15 embryo. The infected embryo was fixed at HH stage 26. (B) The embryo presented in A was stained in whole mount with X-gal to visualize infected cells and their progeny (blue-stained cells). Arrows indicate extra protrusions. Histological sections confirmed that these outgrowths contained β-gal-positive cells (not shown). (C) Virus was injected into the lateral mesoderm adjacent to two consecutive somites within the wing-forming field and two consecutive somites in the flank region of a HH stage 15 embryo. The embryo was examined at HH stage 33. (D) The embryo shown in C was stained with X-gal. Skeletal components in the extra limb presented in C and D were stained in whole mount with Alcian blue. Skeletal elements in wing of control (E) and infected (F) embryos are presented. Long and short arrows in C, D, and F indicate regions of endogenous and induced wings, respectively. Note that an ulna-like structure and four digits were induced within the extra limb (short arrow), and formation of digits in the authentic wing was also affected (long arrow).

patterns restricted to the presumptive limb forming region, we suggest that FGF signaling may play a key role in activating somatic cells to grow from the embryonic body and to form a limb bud.

Outgrowth and patterning of the already developing limb bud is regulated by the reciprocal interactions between the apical ectodermal ridge (AER) and the underlying mesenchymal cells within a progress zone (reviewed by Tabin, 1991). FGF can mimic the AER activities in the growing limb (Niswander *et al.*, 1993; Fallon *et al.*, 1994), interacting with a number of factors (Vogel and Tickle, 1993; Anderson *et al.*, 1993) such as *Sonic hedgehog* (Riddle *et al.*, 1993) in the zone of polarizing activity (ZPA) which regulates pattern formation along an anterior–posterior limb axis. Studies in a chicken mutant, *limbless*, lacking the AER (Carrington and Fallon, 1988) demonstrate that the initial limb budding is independent of the AER activities but that the bud regresses later, similar to the FGF-induced flank outgrowths. Taking these data together, we suggest that FGF can induce budding but that FGF alone is not sufficient for later limb outgrowth and patterning. It remains to be established if co-introduction of FGF with other factors of the AER and/or the ZPA can stabilize and complete both outgrowth and patterning of artificial limbs in the flank region.

We thank Drs. Sakamoto and Terada for cDNA of FGF4, Drs. Niswander, Bader, and Yutzey for their discussion. Our thanks extend to L-L. Ong, L. Cohen-Gould, and L. Miroff for their technical assistance. This work was supported in part by grants from the American Heart Association, the NIH, Mather's Foundation, and the Japanese Ministry of Education, Science and Culture.

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